

## Diverse structural approaches to haem appropriation by pathogenic bacteria

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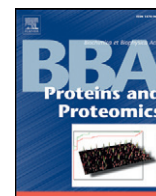
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## Review

## Diverse structural approaches to haem appropriation by pathogenic bacteria

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## ABSTRACT

The critical need for iron presents a challenge for pathogenic bacteria that must survive in an environment bereft of accessible iron due to a natural low bioavailability and their host's nutritional immunity. Appropriating haem, either direct from host haemoproteins or by secreting haem-scavenging haemophores, is one way pathogenic bacteria can overcome this challenge. After capturing their target, haem appropriation systems must remove haem from a high-affinity binding site (on the host haemoprotein or bacterial haemophore) and transfer it to a binding site of lower affinity on a bacterial receptor. Structural information is now available to show how, using a combination of induced structural changes and steric clashes, bacteria are able to extract haem from haemophores, haemopexin and haemoglobin. This review focuses on structural descriptions of these bacterial haem acquisition systems, summarising how they bind haem and their target haemoproteins with particular emphasis on the mechanism of haem extraction.

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## 1. Introduction

Pathogenic bacteria experience a particularly acute difficulty in their requirement for iron. While iron is an abundant element on earth it is insoluble in an oxidizing environment and highly toxic when reduced due to its proclivity to catalyse the formation of reactive oxygen species. All life must overcome these hindrances to satisfy their fundamental requirement for iron. Iron is notably the prime element used for redox chemistry in living organisms, ranging from iron sulphur and haem containing cytochromes of the respiratory chain through iron dependent enzymes in DNA replication and repair to those that catalyse crucial oxygenation and reduction reactions. The problem of low iron bioavailability is further exacerbated for bacteria that must survive within a host environment where, in addition to the natural explanations above, iron abundance is further reduced by the host, both for its own use and as a general defence mechanism against such invaders [1,2].

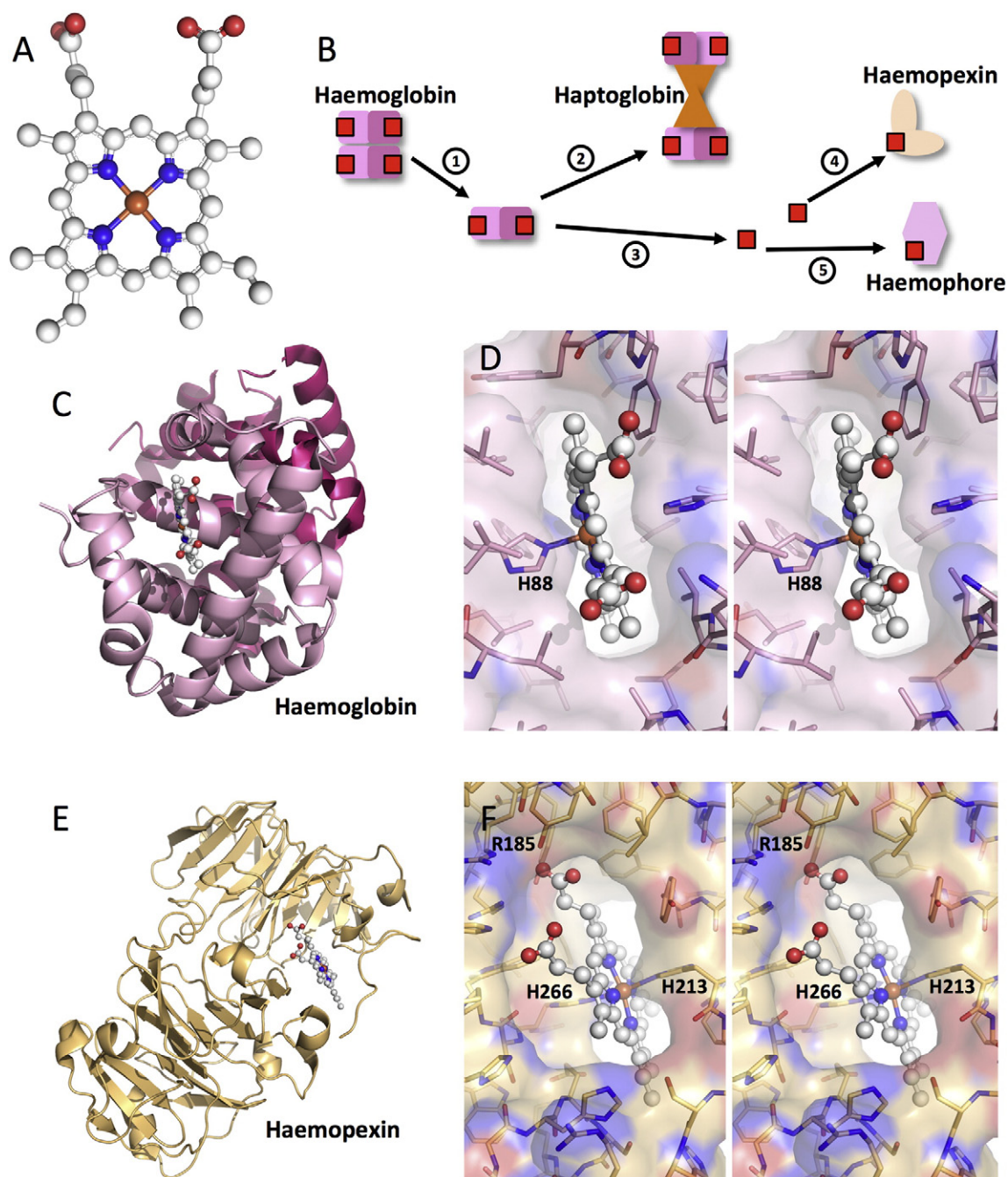
Iron within humans is predominantly confined in haem groups within haemoglobin molecules, accounting for two thirds of total body iron. The second largest reservoir of iron is the ferritin cages, used to store iron inside the cell and regulate intracellular iron levels. Any extracellular iron in serum is quickly bound by transport protein transferrin, which is normally <30% saturated with iron as it circulates in the blood. Transferrin binds iron with incredibly high affinity (association constant of  $10^{20} \text{ M}^{-1}$  [3]), therefore any bacteria seeking to survive systemically, faces the formidable challenge of competing with transferrin or extract iron directly from it. Similarly in other body secretions lactoferrin, acting as part of the innate immune system, binds free iron to inhibit bacteria colonising mucosal surfaces. While many bacteria secrete small

molecules called siderophores that bind to iron with very high affinity and compete with host proteins such as transferrin [4], others extract iron directly from host proteins transferrin and lactoferrin [5] or, as reviewed herein, circumvent the challenge posed by transferrin by importing haem iron, either directly from haemoproteins or by scavenging free haem.

## 2. Host haem sources

The name haem accurately describes a heterocyclic porphyrin ring with a centrally coordinated Ferrous iron ( $\text{Fe}^{\text{II}}$ ) (Fig. 1A). The related haemin molecule (porphyrin ring with Ferric iron ( $\text{Fe}^{\text{III}}$ )) is also often referred to as haem. Haemoproteins bind haem using a combination of occupying one or both of the free iron coordination sites perpendicular to the porphyrin ring (frequently forming covalent bonds), and by interacting with the ring's planar hydrophobic surfaces and sometimes its carboxylate groups. The combination of strong bond formation and water exclusion from the large hydrophobic surfaces leads to very tight interactions. As described by the Heme Protein Database, imidazole groups on histidine side chains are by far the most common protein ligands for coordination of haem iron, with methionine, cysteine and tyrosine side chains used less frequently [6].

While the quantity of haemoglobin-derived iron suggests this would be an attractive iron source for invasive bacteria, the confinement of the vast majority of haemoglobin within erythrocytes renders it unavailable. Spontaneous haemolysis does release small amounts of haemoglobin, with values of up to  $3 \mu\text{M}$  being reported in healthy serum [7], but this is rapidly cleared by the serum protein haptoglobin



**Fig. 1.** Mammalian haem sources. A) Ball-and-stick representation of the haem group coloured by atom (carbon, white; oxygen, red; nitrogen, blue; iron, orange). B) Flow scheme of haem transfer. Following release from red blood cells, haemoglobin dissociates into dimers (1), which are rapidly sequestered by haptoglobin (2). Haem can be released from haemoglobin either spontaneously or as a result of bacterial proteases (3). Serum proteins such as haemopexin rapidly sequester free haem (4), in competition with microbial haemophores (5). C) Cartoon representation of dimeric haemoglobin,  $\alpha$  chain in light pink and  $\beta$  chain in dark pink (PDB: 4XOL [89]). D) Close up of the haem binding site in haemoglobin with a semi-transparent protein surface shown and protein atoms as stick representation. The axial haem ligand is labelled. E) Cartoon representation of mammalian haemopexin (PDB: 1QJS [62]). F) Close-up of the haem-binding site of haemopexin, haem binding side chains mentioned in the text are labelled.

[8] (Fig. 1B). Once released from erythrocytes, haemoglobin tetramers dissociate into dimers that are bound almost irreversibly by haptoglobin and cleared through macrophages [8,9]. As well as facilitating haemoglobin clearance, haptoglobin acts to protect the body from oxidative damage that could otherwise arise from free haemoglobin [10]. This low level of cell-free haemoglobin and haemoglobin-haptoglobin complexes represents a useable reservoir of iron that can be exploited by certain bacteria and levels are likely increased by haemolytic pathogens and during sepsis. However, haemoglobin-bound haem is still far from a readily available iron source, bound as it is by haemoglobin with subpicomolar affinity [11]. A similar challenge is posed by iron

extraction from transferrin, but here the host protein has evolved as an iron transport molecule and therefore must be able to release iron to host cells in response to acidification after internalisation; a bacteria able to exploit transferrin's propensity to open under certain conditions will be able to access the stored iron. While this is no trivial feat, it is rationally simpler than extracting haem from haemoglobin, a protein inherently folded around and fundamentally reliant on its cofactor (Fig. 1C). A histidine side chain of haemoglobin (His87 in  $\alpha$ - and His92 in  $\beta$ -haemoglobin) interacts with one of the free coordination sites of the ferrous iron in haem (Fig. 1D), and the other site coordinates oxygen (in oxy-haemoglobin) or is unbound (in deoxy-haemoglobin). The



occupancy of this second site affects the quaternary structure of haemoglobin, which is also affected by the binding of haptoglobin or oxidation of the iron centre in methaemoglobin. For maximum efficiency, a bacterial system utilising haem from haemoglobin would be able to recognize all structural states.

Serum proteins, principally haemopexin and also albumin, rapidly sequester any free haem to facilitate its clearance and to protect from haem mediated oxidative damage (Fig. 1B). In addition to targeting haemoglobin-derived haem, some bacteria use haemopexin as a haem source, or secrete haemophores to compete with haemopexin in scavenging free haem at very low concentrations (Fig. 1B). The haemopexin structure comprises two  $\beta$ -propeller domains with haem coordination occurring at the interface between the domains (Fig. 1E). Histidine side chains from the inter domain linker and from the C-terminal domain act as axial ligands (Fig. 1F), two arginine and another histidine side chain neutralize the negative charge of the porphyrin carboxylates and multiple hydrophobic side chains from both domains complete the interface. This coordination binds haem with subpicomolar affinity [12].

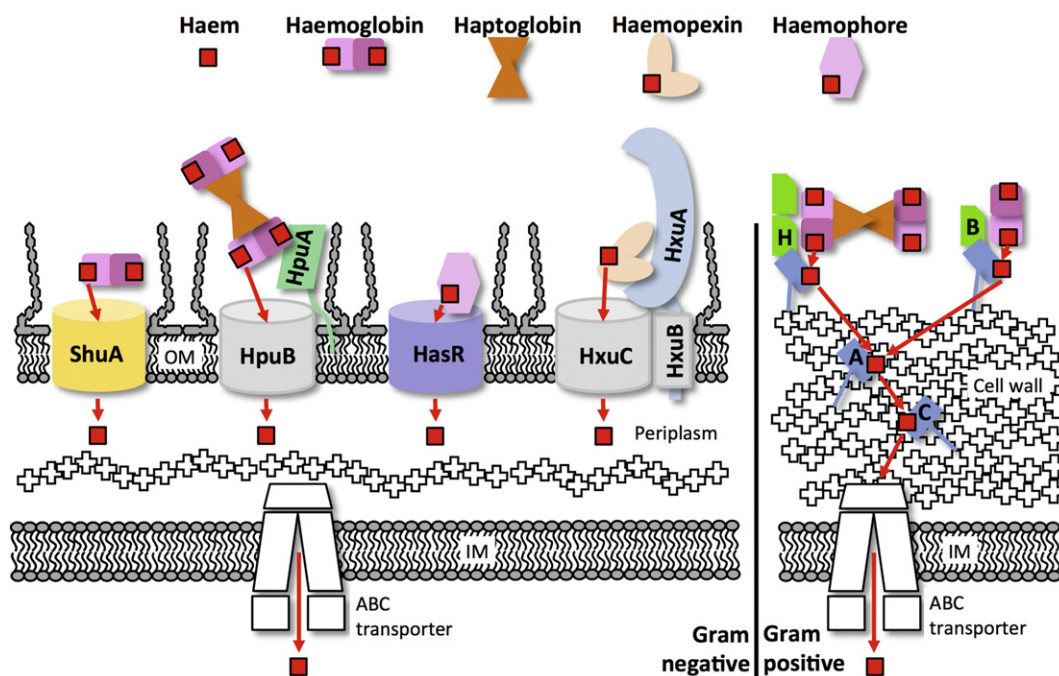
### 3. Bacterial haem import mechanisms

The low availability of extracellular haem necessitates the use of active transport systems for its uptake (Fig. 2). To import through the cytoplasmic membranes of both Gram-positive and Gram-negative bacteria ABC transporters are typically used. These include a haem binding protein outside the membrane, an intra membrane channel and a cytoplasmic ATPase to provide energy. For transport across the outer membranes of Gram-negative bacteria, where no ion gradient or chemical energy sources are available, the TonB system is used. TonB connects a large, 22 stranded outer membrane  $\beta$ -barrel to an inner membrane ExbB/ExbD proton channel to utilise the inner membrane's proton gradient as an energy source for transport across the outer membrane. It is beyond the scope of this review to fully describe the mechanism of ABC transporters and TonB dependent transport, indeed other comprehensive reviews and recent papers cover these subjects [13–15]. Rather, herein consideration is principally given to extracellular events and the diverse systems bacteria have evolved to bind to haem

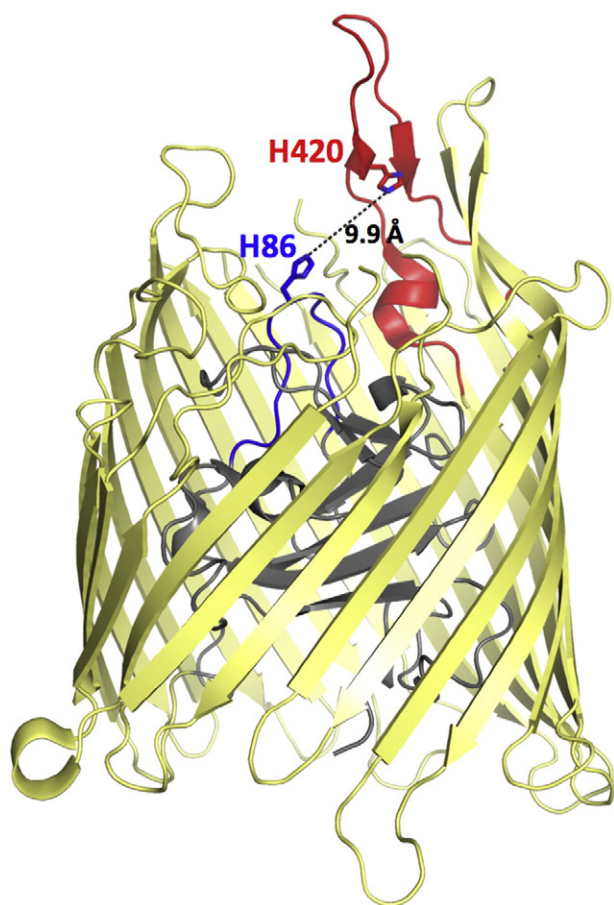
and host haemoproteins in their efforts to secure haem as an iron source. An initial description of single protein receptors is followed by summaries of interesting and more complex multiprotein systems in Gram-negatives, including a siderophore-analogous haemophore system and finally an examination of a fascinating system employed by Gram-positives.

### 4. Monopartite haemoglobin receptors: ShuA of *Shigella*

Arguably the simplest bacterial haemoglobin receptors are found in Gram-negatives and consist of a single outer membrane  $\beta$ -barrel TonB dependent transporter. No structural information is available on how these proteins bind to haemoglobin and extract the haem group, but a protein from the haemorrhagic pathogen *Shigella dysenteriae* has been investigated biochemically and structurally characterised in isolation. Originally identified as able to confer survival on haem as an iron source to *E. coli*, the *Shigella* haem uptake (*shu*) operon contains an outermembrane TonB dependent transporter [16,17]. Haem was demonstrated to transfer from haemoglobin to purified ShuA in stop flow experiments with oxidised methaemoglobin being the preferential haem donor [18]. This is consistent with methaemoglobin being the primary form of haemoglobin available during gastrointestinal bleeding induced by *S. dysenteriae* [19]. Additionally, two conserved histidine residues identified in the ShuA sequence were confirmed as playing a role in the extraction of haem from haemoglobin as a double mutant of His86 and His420 was unable to perform this function [18]. The crystal structure of ShuA revealed, as expected, a classical 22-stranded  $\beta$ -barrel occluded by an N-terminal plug domain [20] (Fig. 3). The two proposed haem ligands His86 and His420 are approximately 10 Å apart (significantly further than the two histidines coordinating haem in haemopexin at 4.3 Å) suggesting structural rearrangement occurs upon haemoglobin or haem binding (Fig. 3). Interestingly, while His420 is located on one of the ShuA extracellular loops (L7), His86 is situated on a loop of the plug domain. This loop was previously demonstrated to contribute to substrate binding in other TonB dependent receptors [21,22] and also to be involved in the extraction of iron from transferrin in the transferrin TonB dependent transporter, TbpA [23].



**Fig. 2.** Graphical summary of the different haem uptake systems employed by pathogenic bacteria and summarised in this review. On the left are Gram-negative systems for which some high-resolution structural information is available and on the right the characterised *Isd* systems of Gram-positives. Components with structures elucidated are coloured with colouring scheme maintained throughout the review, and components for which structural information is missing are grey.



**Fig. 3.** Crystal structure of ShuA. A cartoon representation of the ShuA structure (PDB: 3FHH [20]), with the barrel in pale yellow and the plug domain in grey. The proposed haem binding ligands are labelled and the loops featuring them are coloured red and blue.

Other monopartite haemoglobin receptors have also been identified; such as HmbR in *Neisseria* [24] and HemR in *Yersinia* [25] but the structural basis of haemoprotein binding by all of these receptors remains undetermined. The rest of this review focuses on multiprotein haem uptake systems where the structural mechanisms of haemoprotein binding or haem transfer have been identified.

### 5. Bipartite haemoglobin receptors: the HpuA/B system of *Neisseria*

The HpuA/B system is conserved throughout the *Neisseriaceae* family of beta proteobacteria including in the human pathogens *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Kingella* species and *Eikenella corrodens*. HpuA/B has been shown to bind to haemoglobin and to haptoglobin-haemoglobin complexes and extract haem directly from them [26–28]. Like the ShuA system, the HpuA/B system uses a TonB dependent transporter (HpuB) to facilitate iron transport across the outer membrane. The second protein, HpuA, does not act as a haemophore but is rather a lipoprotein attached to the outer surface of the bacteria and acts in a somewhat enigmatic, and yet essential way to enable haem uptake [28–30].

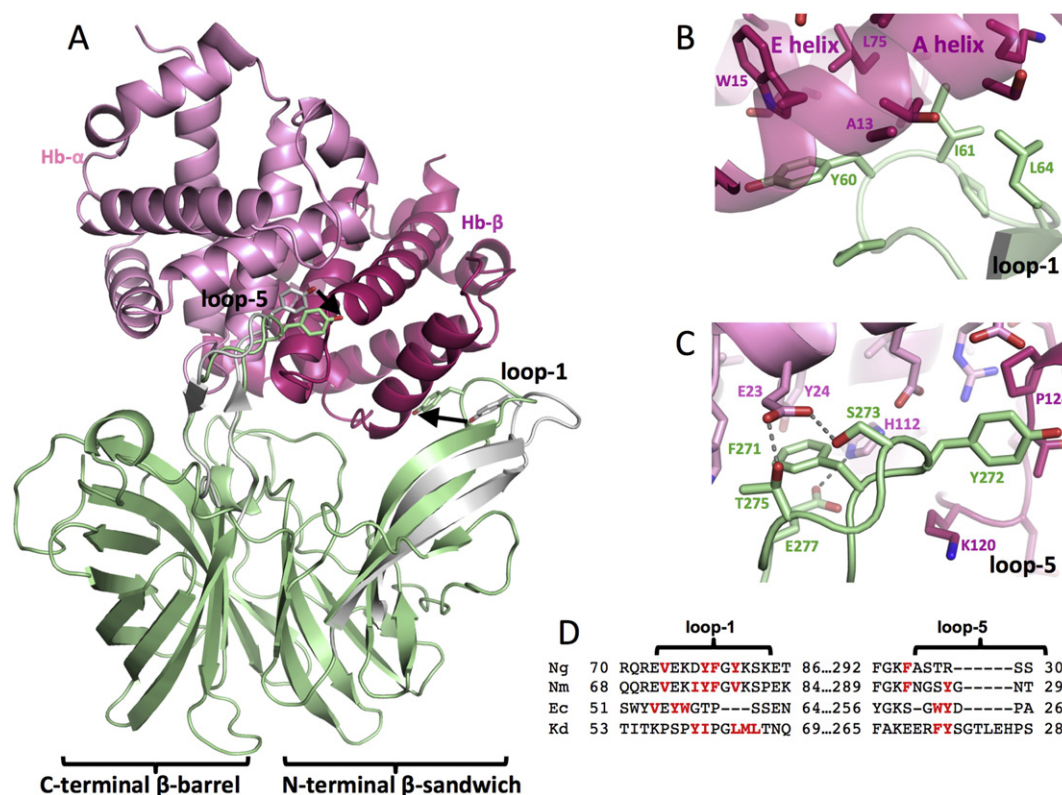
The essentiality of HpuA both for bacterial survival on haemoglobin as a sole iron source and for the high affinity binding to haemoglobin at the cell surface in combination with HpuB were demonstrated showing HpuA is required for the uptake of haem from haemoglobin [27,28]. However, initially no direct observation of a HpuA-haemoglobin interaction on the surface of cells was observed [28]. This changed last year when the structural basis for haemoglobin binding to *Kingella denitrificans* HpuA was described [31]. The structure of HpuA is

reminiscent of other extracellular lipoproteins of *Neisseriaceae* with a single domain comprising a C-terminal small  $\beta$ -barrel and an N-terminal loose  $\beta$ -sandwich (Fig. 4A). Other *Neisseria* lipoproteins that bind to host proteins share the same fold, including the transferrin binding proteins TbpB and the Factor H binding protein; however, all three proteins bind their targets using different interfaces. In the case of HpuA, two long loops extend from the core of the fold and expose hydrophobic residues that interact with hydrophobic areas exposed on the surface of haemoglobin. In comparing the free and haemoglobin-bound HpuA structures, movement is limited to these HpuA-loops (referred to as loop-1 and loop-5) as they move in a slight pincer movement to lock onto the haemoglobin surface (Fig. 4A). Loop-1 inserts between the A and E helices of the haemoglobin  $\beta$  chain presenting the side chains of Tyr60 and Ile61 to interact with Trp15 and Leu75 (Fig. 4B). Loop-5 on the other hand, binds at the haemoglobin  $\alpha$ - $\beta$  chain interface with side chains of Phe271 and Tyr272 burying hydrophobic surfaces and Glu277 forming a solitary salt bridge with His112 of the haemoglobin  $\alpha$  chain (Fig. 4C). There are other minor contributions from shorter HpuA loops. No significant changes in the haemoglobin structure are observed on HpuA binding.

The HpuA-haemoglobin interaction is relatively weak with a dissociation constant of 6  $\mu$ M, perhaps explaining the failure to detect this on the surface of cells and also suggesting that the role of HpuA is not to accomplish the initial capture of haemoglobin at the surface of the cell, but rather to act in conjunction with HpuB to form a complete high-affinity receptor. There are also reports that HpuA is involved more directly in the mechanism of haem uptake and/or in subsequent haemoglobin release [28]. However, there is no suggestion from the structure of the complex with HpuA that haemoglobin loosens its grip on haem as a result of being captured [31], therefore we currently have no understanding of the structural basis for these proposed roles.

Interestingly, although HpuA from diverse *Neisseriaceae* were observed to bind haemoglobin in a pull down assay, the critical haemoglobin-binding residues of *K. denitrificans* HpuA are fairly poorly conserved. Indeed the two long haemoglobin-interacting loops lack the levels of conservation that would be expected for functionally important regions and were previously shown to be subject to variation in response to immune selection [32]. Importantly, and unusually for solvent exposed loops, these loops do always contain hydrophobic residues, which are assumed to interact with the same pockets on the surface of haemoglobin as observed for *K. denitrificans* HpuA (Fig. 4D). Indeed, it seems likely that variation of these long exposed surface loops is driven by immune selection but that conservation of hydrophobic residues is sufficient to ensure maintenance of the haemoglobin-binding function.

In lieu of structural information of HpuB, much of our assumed structural understanding for the mechanism of HpuB dependent haem import is derived from studies of the related transferrin receptor TbpA, with which it shares higher similarity than with ShuA. However, the substrate structure and challenge of iron/haem extraction by the two systems is significantly different. A model of the TonB-dependent receptor HpuB has been constructed using the transferrin receptor TbpA as a template [32]. Sharing about 30% identity, the TbpA based HpuB model is likely to be essentially accurate in the description of the plug domains and the transmembrane beta strands. While, the extracellular loops, likely to be involved in stripping haem from haemoglobin, show little conservation between the two proteins, one important structural feature of TbpA, the long loop extending outwards from the plug domain, is predicted to be of similar length in HpuB. This loop was implicated in TbpA function [33,34] and later observed to interact directly with human transferrin to contribute to opening its iron-binding site [23]. While the plug loop (and indeed the whole of the plug domain) is well conserved in HpuB homologues, it remains to be seen if it fulfils a similar function in HpuB as in TbpA. In contrast to ShuA, no histidines in HpuB are fully conserved, either on this plug loop or the extracellular loops, impeding prediction of a haem binding site.



**Fig. 4.** Haemoglobin capture by HpuA. A) Cartoon representation of haemoglobin (pink) captured by HpuA (green) superposed on the structure of HpuA alone (white). The two loops critical for the interaction are labelled and their pincer movement indicated (PDB: 5EE4 [31]). B) Close up view of the interactions between HpuA loop-1 and haemoglobin β chain with interacting hydrophobic residues shown as sticks. C) Close up view of the interactions between HpuA loop-5 and the α and β chains of haemoglobin. Important side chains are indicated and hydrogen bonds shown as black dashed lines. D) Sequence alignment showing variation of the loop regions of HpuA from *N. gonorrhoeae* (Ng), *N. meningitidis* (Nm), *E. corrodens* (Ec) and *K. denitrificans* (Kd). Hydrophobic residues on the loops are coloured red.

## 6. Haemophores: the HasA/HasR and HmuY/HmuR systems

HasA was identified as an iron regulated 19 kDa secreted protein of *Serratia marcescens* that binds directly to haem and is essential for growth on haem or haemoglobin as a sole iron source [35]. Preincubating HasA with haemoglobin was observed to inhibit haem binding by the bacterial protein, suggesting it was able to extract haem from the host protein *in vitro*. Subsequent analysis of HasA using biochemistry and biophysics revealed it bound one molecule of haem per HasA with a dissociation constant of 18 pM [36,37].

Structural characterisation of HasA from *S. marcescens* followed, revealing a protein with a six-stranded antiparallel β-sheet packed against four α-helices [38] (Fig. 5A). Two strands of the sheet (β4 and β5) are significantly curved and the loop connecting them forms a fundamental part of the haem-binding site. The warped nature of this part of the sheet combined with a similar curvature of the loop between β2 and β3 constitutes one side of the haem-binding pocket, with the other side composed primarily of residues in a loop preceding β2 including a 3<sub>10</sub> helix (η2). The iron coordination sites perpendicular to the porphyrin ring are occupied by the phenolate group of a deprotonated tyrosine (Tyr75) side chain at the end of β4 and a histidine (His32) side chain of the loop region preceding β2 (Fig. 5A). Tyr75 additionally interacts with His83, a side chain found to be important for high affinity haem binding as it donates a hydrogen bond to the phenolate group, stabilising it [37, 39]. It was also suggested that in a mutant with Tyr75 mutated to alanine (Y75A), His83 could substitute for Tyr75 as the axial ligand [40]. The solution structure of apo HasA revealed the His32 loop to exist in a strikingly different conformation in the absence of haem, moving up to 30 Å into an open form [41]. There are additional hydrophobic interactions involving side chains from the aforementioned regions and from

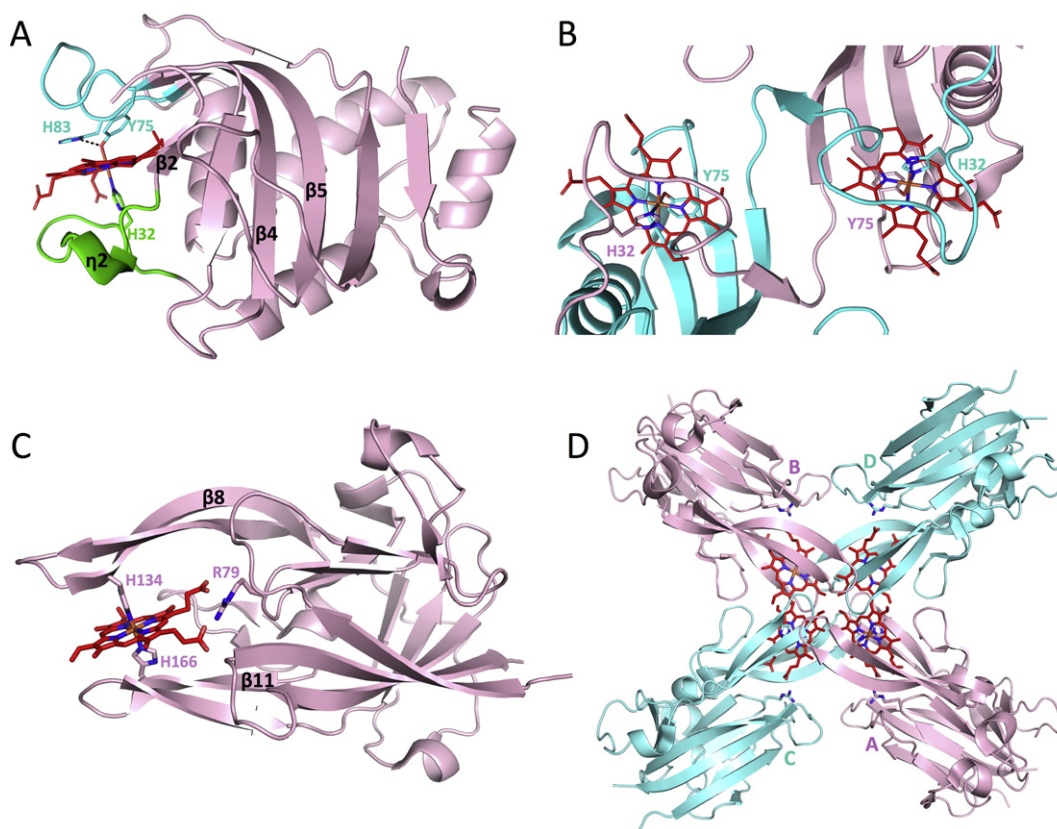
the underlying α2 helix creating a tight-binding binding pocket. The His-Tyr coordination of the haem moiety contributes to a lower redox potential of the bound iron and favours ferric iron haemin over ferrous haem. Further work identified and structurally characterised a dimeric form of HasA with two monomers interlocked by a domain swap of the N-terminal 48 amino acids, such that the haem groups were coordinated between Tyr75 of one monomer and His32 of the second [42] (Fig. 5B). Although this dimeric form of HasA was unable to pass Haem onto HasR, it could pass the haem onto monomeric HasA and the authors postulated the dimeric form could act as a haem reservoir for *S. marcescens*.

HasA/HasR systems are not limited to *S. marcescens* and have also been identified in *Pseudomonas aeruginosa* and *Yersinia* species [43]. Surprisingly, despite high overall sequence similarity (>50% between homologs from all three genera), *Yersinia* HasA homologs do not conserve His32, rather containing glutamine at this position. The crystal structure of the *Y. pestis* and *Y. pseudotuberculosis* HasA proteins revealed the glutamine does not contribute to haem binding and that the axial coordination site occupied by His32 in *S. marcescens* HasA remains unoccupied [44,45]. Rather, two arginine side chains Arg40 and Arg114 stack against this haem surface.

Despite this large amount of work going into defining how HasA binds to haem, there has been no description of how HasA can extract haem directly from haemoglobin and it is possible it rather competes with host haem-scavenging proteins for free haem.

A structurally distinct hemophore called HmuY has been characterised from *Porphyromonas gingivalis* [46]. HmuY was characterised biochemically and structurally and found to shift from a dimeric form in its apo state to a tetramer when bound to haem [47]. Haem binding was found to be dependent on two histidine sidechains (His134 and His166) and





**Fig. 5.** Haem binding by HasA and HmuY. A) Haemophore HasA shown as cartoon representation in pink with the loops containing haem ligands coloured green and cyan. Side chains that form key interactions are shown as sticks and the bound haem moiety as red sticks (PDB: 1B2V [38]). B) The HasA domain swapped dimer storing two haem groups (PDB: 2CN4 [42]). C) The structure of a HmuY monomer (pink cartoon) showing the haem group (red sticks) and haem binding residues (pink sticks) (PDB 3H8T [49]). D) The cross-shaped tetrameric structure of holo HmuY with chains A and B coloured pink and chains C and D in cyan (PDB 3H8T [49]).

the bound haem iron is in a hexa-coordinate environment [48]. The crystal structure of the tetrameric holo HmuY was solved to reveal a novel all- $\beta$  fold resembling a right hand (Fig. 5C and D) [49]. Similar to HasA, the haem group in HmuY is sandwiched between two secondary structure elements at the edge of the folded domain, but here it is two histidine side chains on the “thumb” and “ring finger” strands (His134 on  $\beta 8$  and His166 on  $\beta 11$ , respectively) that coordinate the haem iron. In addition, HmuY makes polar interactions with the propionate groups of haem, including a salt bridge from the side chain of Arg79. The haem-dependent tetramer forms a cross shape with four haem molecules buried in the core of the protein and hence hidden from other potential haem binding proteins.

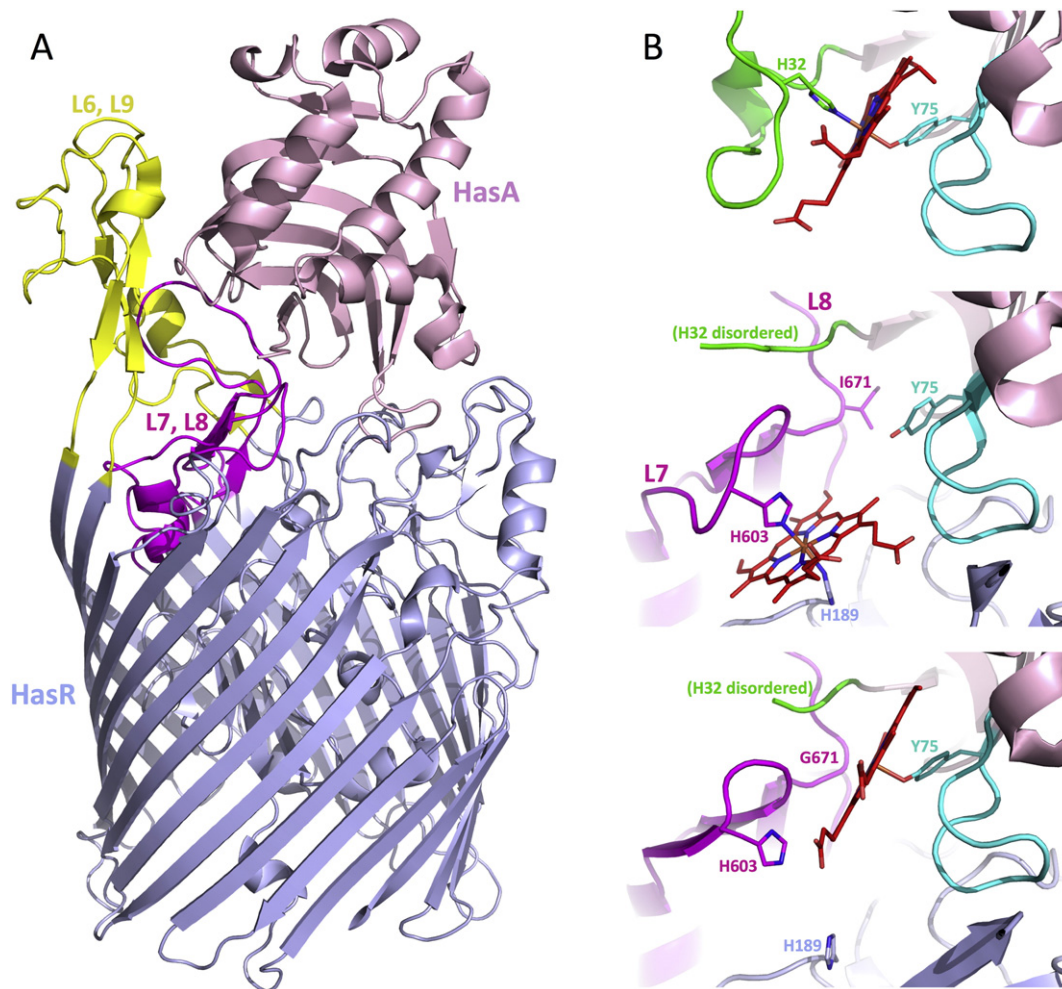
Interestingly, as opposed to extracting haem from intact host haemoproteins, *P. gingivalis* secretes proteases that degrade haemoglobin, haptoglobin and haemopexin and thereby release haem for HmuY scavenging [50]. Critically for this appropriation strategy, HmuY is resistant to proteolysis by these secreted proteases [49].

The other essential component of any haemophore system is the haemophore receptor; in the case of HasA this protein is called HasR (and for HmuY it is HmuR). HasR itself is able to bind haem but at a much lower affinity than HasA (200 nM  $K_d$  for HasR compared to 18 pM for HasA) [51]. In spite of this lower affinity, haem spontaneously transfers from HasA to HasR [51]. A direct interaction between HasR and HasA has been observed with an apparent  $K_d$  of 7 nM and this was not dependent on the haem bound state of HasA, as apoHasA bound with the same affinity as holoHasA [52]. HasR is able to import free haem as well as haem from HasA and is a typical TonB dependent receptor comprising a  $\beta$ -barrel closed by an N-terminal plug domain and with an N-terminal extension including the Ton box to bind TonB [53,54].

The breakthrough in describing the mechanism of haem transfer from HasA to HasR came with the publication of a series of structures of the complex between the two proteins [54] (Fig. 6). HasR resembles other TonB dependent transporters and contains long loops between beta-strands on the extracellular side. Two long extracellular loops (L6 and L9, coloured yellow in Fig. 6A) form the main points of contact in binding to HasA, while two other loops (L7 and L8, coloured magenta in Fig. 6) are critical in the transfer of haem from its high affinity site on HasA to the much weaker binding site on HasR. The L7 loop of HasR sterically clashes with the His32-containing loop of HasA causing it to be disordered and thereby liberating one side of the haem binding pocket and removing the His32-mediated haem-iron coordination (Fig. 6B). This in itself is insufficient to release the haem group, which also requires the side chain of Ile671 on the L8 loop of HasR. In the HasR-HasA complex, Ile671 occupies the same position relative to HasA as the haem group does in the HasA-haem complex structure, and therefore sterically triggers the expulsion of haem from this site. This combination of events was neatly deciphered by resolving crystal structures of the complexes between the wild type HasA with wild type and I671G mutant HasR. The I671G mutant HasR was able to free up haem from coordination by HasA His32 but not to completely eject it from its HasA binding site (Fig. 6B lower panel).

The haem binding site on HasR can also be observed in the HasA-HasR structure and involves histidine side chains from the plug loop and extracellular loop-7. Indeed, this binding mode of haem is likely to be very similar in the haemoglobin receptor ShuA, whose haem binding histidines are located on the same features.

Structural information on HmuR and on the transfer of haem from HmuY to HmuR is not yet available. Presumably prior to displacing



**Fig. 6.** Haem transfer from HasA to HasR. A) View of HasA (pink) captured by HasR (light blue) (PDB: 3CSL [54]). Extracellular loops of HasR important for HasA binding (L6 and L9) are coloured in yellow and those involved in extracting haem (L7 and L8) in magenta. B) Aligned views showing transfer of haem from HasA to HasR (PDB: 1B2V (upper), 3CSL (middle), 3DDR (lower) [38,54]). HasR loop L7 displaces the His32 containing HasA loop (middle and lower panels) to disrupt haem binding by HasA (upper panel). Also essential for haem removal is the steric clash between Ile671 of HasR and the haem group itself as the I671G mutant structure (lower panel) fails to complete haem transfer from its HasA to HasR binding sites.

HmuY-bound haem, HmuR must induce the dissociation of the haemophore tetramer.

## 7. Haemopexin receptor: the HxuA/B/C system of *Haemophilus*

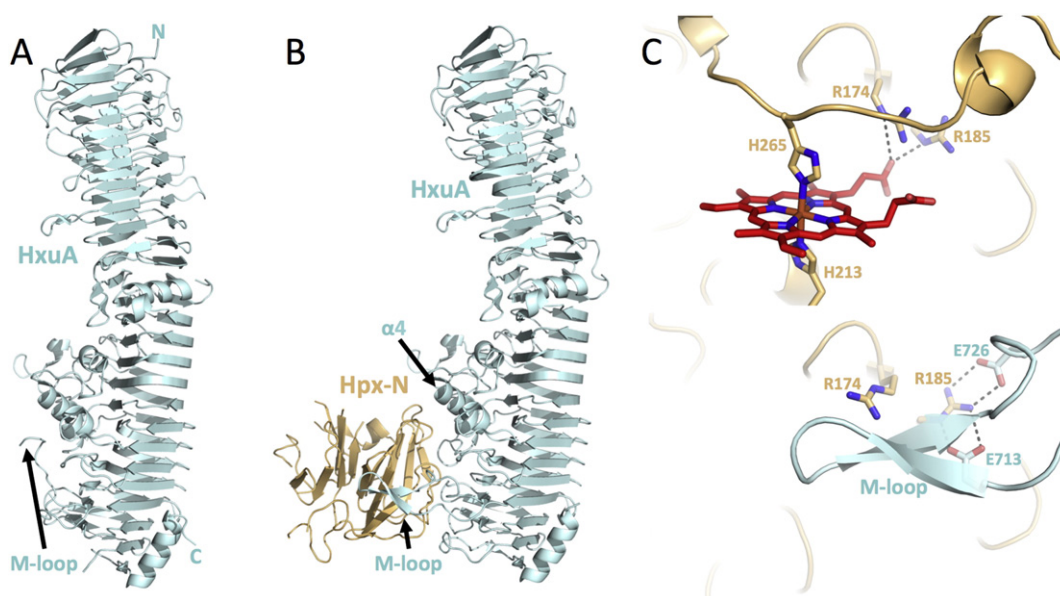
As mentioned in the introduction, haemoglobin is not the only potential source of haem for bacteria that colonise higher eukaryotes. *Haemophilus influenzae* is able to extract the haem group from the host scavenging protein haemopexin. The *Hxu* operon encoding genes for the expression of HxuA/HxuB/HxuC proteins was identified to be essential for the use of haem from haemopexin [55–57]. In this system, HxuC encodes the TonB dependent transporter for the uptake of haem across the outer membrane. HxuA, like HpuA is an extracellular protein tethered to the outer membrane, however, in contrast to HpuA, HxuA is not a lipoprotein but is rather secreted as a two-partner secretion system, with HxuB forming the secretion channel and the C-terminal helix of HxuA being anchored within the HxuB barrel lumen via a disulphide bond stabilised C-terminal section of sequence [58]. The role of HxuA is to bind haemopexin, which it does with nanomolar affinity [59], and induce it to release haem for subsequent import by HxuC.

While earlier work had solved the structure of the N-terminal portion of HxuA [60], a more recent structural breakthrough elucidated not only the structure of full length HxuA, but also its complex with a portion of haemopexin, shedding light on the mechanism of haem removal [61] (Fig. 7). HxuA forms a kinked, elongated, right-handed  $\beta$ -

helix with six  $\alpha$ -helices clustered around the C-terminal region of the  $\beta$ -helix (Fig. 7A). This C-terminal end of the protein is the functional end, interacting with haemopexin, while the N-terminal end is the secretion domain resembling classical two-partner secretion proteins. Meanwhile, it was shown that the N-terminal  $\beta$ -propeller domain of haemopexin is primarily responsible for the interaction with HxuA, as although the N-terminal domain showed a weaker interaction than the full-length protein, there was no interaction detected between HxuA and the haemopexin C-terminal  $\beta$ -propeller domain [61].

Very little structural alteration of HxuA is observed upon binding to the haemopexin N-terminal domain, only the M-loop (moving loop) shows a significant shift, changing from a partially disordered state in apo HxuA to a short  $\beta$ -hairpin in the haemopexin bound structure. An extensive interface ( $\sim 1600 \text{ \AA}^2$ ) is buried between the two proteins and  $\alpha$ -helix 4 and the adjacent part of the  $\beta$ -helix of HxuA are critical in forming the bulk of the interface. Ionic and polar interactions dominate with HxuA providing a complementary charged surface to haemopexin. The M-loop is also involved in the interactions and is fundamental in facilitating haem removal through two mechanisms. Firstly, when comparing the HxuA bound and haem bound haemopexin structures, the HxuA M-loop occupies the same space as both the linker between the C- and N-terminal domains of haemopexin (which contains the haem iron ligand His265) and the haem group itself (Fig. 7C). Secondly, the M-loop also coordinates the haemopexin side chain Arg185, which natively interacts with the haem group via one of its propionate





**Fig. 7.** Haemopexin binding and haem removal by HxuA. A) The HxuA structure with partially disordered M-loop indicated (PDB: 4RM6 [61]). B) Haemopexin (light orange) bound to HxuA (pale cyan) showing the reordered M-loop (PDB: 4RT6 [61]). C) Aligned views of the haem-binding site in haemopexin alone (upper panel, PDB: 1QJS [62]) and in the haemopexin-HxuA complex (lower panel, PDB: 4RT6 [61]). The M-loop of HxuA disrupts the haemopexin haem ligands His265 and Arg185 to displace the haem.

groups (Figs. 7C and 1F). In addition to the M-loop effect, loops of the HxuA  $\beta$ -helix and  $\alpha$ -helix cluster would clash with the position of the C-terminal  $\beta$ -propeller. Interestingly, although the M-loop was essential for the utilisation of haemopexin-derived haem, it is not essential for haemopexin binding. Together these observations suggest that after initial binding to HxuA, the inter domain interactions of haemopexin are disrupted opening up the protein and that insertion of the M-loop at this domain interface liberates the haem group that normally resides therein. In fact, the authors of the original haemopexin structure envisaged such a mechanism of haem release upon noting the labile nature of His213 on the inter domain linker and the role a 'cushion' of water molecules played at the domain interface [62].

Questions remain over the recycling of the Hxu system and addition potential roles played by the membrane receptor HxuC. A direct interaction between HxuA (or HxuA-haemopexin) and HxuC is assumed from the essential nature of the HxuA membrane anchor. Is this interaction intimate enough to ensure complete transfer of the haem after release? Does HxuC play a role in releasing haemopexin from HxuA following haem extraction? Such a recycling would be required for efficient function and yet HxuA clearly binds to apo haemopexin. Future structural investigation of the entire complex will be required to answer these questions.

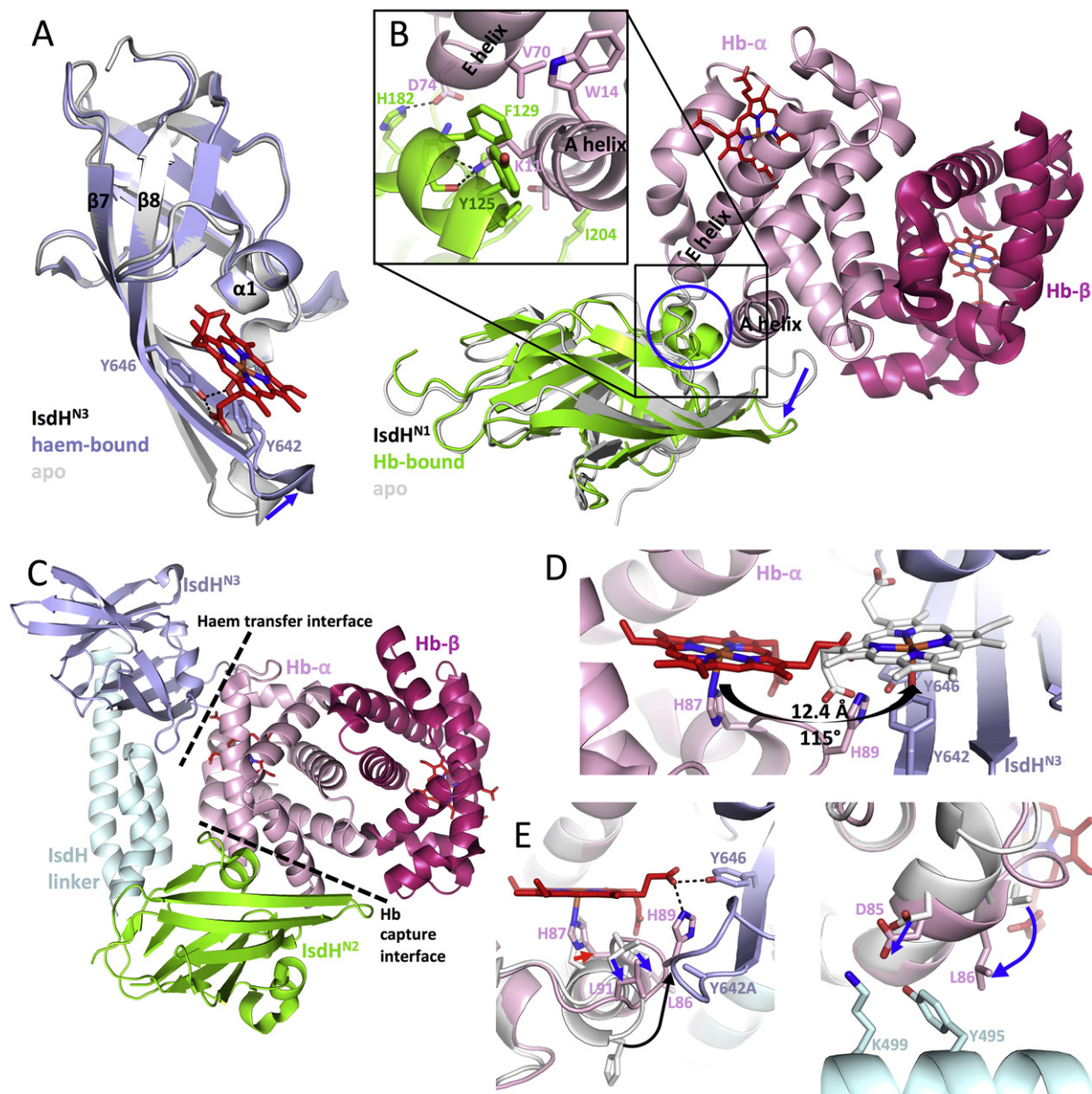
## 8. Gram-positives: the IsdA-I system of *Staphylococcus*

Gram-positive bacteria of the phylum *Firmicutes* use haem uptake systems that make use of near-iron transporter (NEAT) domains. The best characterised of these is the iron-regulated surface determinant (Isd) system of *Staphylococcus aureus* [63], comprising nine proteins: IsdA through IsdI. Outside the cell, IsdH and IsdB are implicated in haem extraction from haemoglobin and transfer haem through the cell wall via IsdA and IsdC [64–66] to the plasma membrane ABC transporter complex formed by IsdD, IsdE and IsdF (Fig. 2) [67]. Once inside the cell, iron is liberated from the haem by two intracellular mono-oxygenase proteins, IsdG and IsdI [68].

As mentioned previously, this review focuses on structural aspects of extracellular events, in this case the NEAT domain containing IsdA, IsdB, IsdC and in particular, IsdH. Single NEAT domains are found in IsdA and IsdC, while IsdB and IsdH contain two and three NEAT domains respectively [69]. NEAT domains are divided into four types based on their

primary amino acid sequences. The first NEAT domain of IsdB (IsdB<sup>N1</sup>) and the first two of IsdH (IsdH<sup>N1</sup> and IsdH<sup>N2</sup>) fall into type I and the final domains of these two proteins (IsdB<sup>N2</sup> and IsdH<sup>N3</sup>) belong to type II. The NEAT domains of IsdC and IsdA are categorized as type III and type IV respectively. While NEAT domain types II, III and IV all bind haem with dissociation constants reportedly varying from 30 nM to sub nanomolar [65,70–74], type I domains do not, but rather bind to haemoglobin [71,75,76]. Structural information of each type of NEAT domains has been published, revealing a shared  $\beta$ -sandwich fold (Fig. 8A) [67,69,77–81]. The structural basis of haem binding to NEAT domain types II, III and IV has also been shown, with the types sharing a binding site at one end of the protein formed from the long  $\beta$ -strands  $\beta$ 7 and  $\beta$ 8 on one side and an  $\alpha$ -helix ( $\alpha$ 1 in IsdH<sup>N3</sup>) on the other (Fig. 8A) [67,77,79]. A tyrosine side chain (Tyr642 in IsdH<sup>N3</sup>), which is conserved in haem-binding type II, III and IV NEAT domains, acts as a single axial ligand for the haem iron. A second conserved tyrosine side chain (Tyr646 in IsdH<sup>N3</sup>) stacks against one of the haem pyrroles and forms two important hydrogen bonds, donating to the haem propionate and accepting from the haem-binding tyrosine hydroxyl group enhancing the nucleophilic nature of the haem ligand (Fig. 8A).

The structural basis of haemoglobin capture by type I NEAT domains has also been elucidated [76,82–84]. No significant structural changes to haemoglobin are observed upon IsdH<sup>N1</sup> binding, but notable movements of components of the NEAT domain occur (Fig. 8B). Haemoglobin interacting residues are predominantly on loop 2 of IsdH<sup>N1</sup>, which converts to an  $\alpha$ -helix upon binding to haemoglobin (blue circle in Fig. 8B) and presents hydrophobic side chains (including Tyr125 and Phe129) to interact with the haemoglobin surface (Fig. 8B). The IsdH<sup>N1</sup> binding site on haemoglobin is distant from the haem binding site and involves the A and E helices of  $\alpha$  haemoglobin (Fig. 8B). Contributions to haemoglobin binding are also made by other IsdH<sup>N1</sup> loops including loop-8, which moves significantly upon haemoglobin binding (blue arrow in Fig. 8B) and includes the Ile204 side chain that interacts hydrophobically with the other side of the A helix of  $\alpha$  haemoglobin, and loop-6, which includes the salt-bridge-forming His182 side chain. Additionally, a negatively charged pocket on the surface of IsdH<sup>N1</sup> is created to accommodate the side chain of haemoglobin  $\alpha$  residue Lys11. The interaction between type I NEAT domains and haemoglobin is relatively tight, with a 5–100 nM dissociation constant and is not affected by the oxygenation or ligand-bound state of haemoglobin [71,76,85]. Size



**Fig. 8.** Haem extraction from haemoglobin by IsdH. A) Haem binding by the type II NEAT domain IsdH<sup>N3</sup>. Haem bound IsdH<sup>N3</sup> is shown as a blue cartoon (PDB: 2Z6F [79]) and apo IsdH<sup>N3</sup> as white cartoon (PDB: 2E7D [79]). The haem binding residues are indicated and the protein dynamics upon binding indicated by the blue arrow. B) Haemoglobin binding by the type I NEAT domain IsdH<sup>N1</sup>. Apo IsdH<sup>N1</sup> (PDB: 2H3K [69]) is shown as white cartoon representation superposed on haemoglobin-bound IsdH<sup>N1</sup> (PDB: 3SZK [76]) as green cartoon. Haemoglobin chains are in different shades of pink and protein dynamics are indicated by the blue arrow and circle. Inset panel shows the detail at the interface with interacting side chains labelled and hydrogen bonds shown as black dashes. C) Overall view of the IsdH<sup>N2-3</sup>-haemoglobin complex (PDB: 4XS0 [84]) with colours conserved from panels A) and B) and the IsdH linker domain in pale cyan. D) The modelled movement of the haem group from its binding site on haemoglobin (red sticks, (PDB: 4XS0 [84])) to that on IsdH<sup>N3</sup> (white sticks, PDB: 2Z6F [79]). E) Induced movements of haemoglobin upon binding to IsdH<sup>N3</sup> (left panel) and IsdH linker domain (right panel). Haemoglobin alone is shown in white (PDB: 1HHO [90]) and haemoglobin bound to IsdH<sup>N2-3</sup> is pink (PDB: 4XS0 [84]). Side chains are shown as labelled sticks and the dynamics indicated by blue arrows.

exclusion chromatography and isothermal titration calorimetry results suggest the N1 domain of IsdH and IsdB interactions are specific for the haemoglobin  $\alpha$  chain, while IsdH<sup>N2</sup> will also bind haemoglobin  $\beta$ , but with reduced affinity [76].

The interaction between the haem-binding type II NEAT domain IsdH<sup>N3</sup> and haemoglobin has also been structurally characterised in the context of a longer IsdH<sup>N2-N3</sup> construct with the haem binding Tyr642 residue mutated to prevent the stripping of haem from haemoglobin and thereby maintaining the structural integrity of haemoglobin [82]. More recently, a higher resolution structure was obtained by additionally mutating the loop 2 helix of the IsdH<sup>N2</sup> domain of this construct to that of the IsdH<sup>N1</sup> domain, ensuring specificity for haemoglobin  $\alpha$  chain and thereby improving monodispersity of the

complex [84]. This structure can be thought of as an intermediate state, where haemoglobin has been captured and positioned for haem removal. As such, it offers a tantalising glimpse as to one mechanism of haem appropriation from haemoglobin. The N2 and N3 domains of IsdH are linked by a short helical bundle domain [73] and form a dumb-bell shaped protein to interact with haemoglobin at two different sites (Fig. 8C) [82,84]. The N2 domain interactions with haemoglobin are highly similar to those observed previously for the N1 domain and concern the A and E helices of haemoglobin and loop 2 of IsdH<sup>N2</sup>. This interface is termed the haemoglobin capture interface, while that between the IsdH<sup>N3</sup> domain and haemoglobin is called the haem transfer interface. At the haem transfer interface the transfer of the haem group between its binding sites on haemoglobin and IsdH<sup>N3</sup> can be visualised



by superposing the haem-bound LsdH<sup>N3</sup> structure onto that of the haemoglobin-LsdH<sup>N2-N3</sup> Y642A structure (Fig. 8D). The two binding sites accommodate haem molecules in coplanar orientations, signifying the haem molecule need not rotate perpendicular to the porphyrin plane, but rather shifts by ~12 Å and rotates ~115° in this plane [82,84].

Binding to LsdH<sup>N2-N3</sup> causes significant rearrangement of haemoglobin at the haem-binding pocket, principally localised on helix F, which contains the axial haem ligand His87. The His87 side chain still coordinates haem but its Cα and Cβ atoms move significantly (red arrow Fig. 8E) potentially weakening the interaction. Further significant movement is seen of the side chains of Leu86 and Leu91 (blue arrows Fig. 8E), opening up the haem-binding pocket, which natively incorporates hydrophobic interactions between these and the porphyrin ring system. Combined, these alterations likely dramatically reduce the affinity of haemoglobin for haem. It is not immediately apparent how the structural alteration in haemoglobin is instigated, but it is probable that, at least in part, interactions with the helical linker domain of LsdH are responsible. The side chain of LsdH Tyr495 provides hydrophobic interactions for the relocated haemoglobin Leu86 side chain and LsdH Lys499 likely influences the position of haemoglobin Asp85 through electrostatic interactions (Fig. 8E).

## 9. Summary and remaining questions

The low availability of free haem and tight haem-binding by mammalian scavenging proteins pose a significant hindrance to haem uptake by bacteria living inside such hosts. To overcome this, bacteria employ different strategies, either secreting haemophores to compete with, extracting the haem direct from, or secreting proteases to degrade host haemoproteins (Fig. 2). A wide variation exists within systems extracting haem direct from host proteins, with the simplest consisting of single protein TonB dependent receptors and more complicated systems utilising a second protein (e.g. HpuA), protein complex (e.g. HxuA/B) or a multi-protein haem transport chain (LsdH/B/C/A). It is interesting to compare the mechanisms of capture of haemoproteins, which reveals the adaptation of individual systems for their targets. Structures of two haemoglobin-receptor complexes have revealed how hydrophobic interactions predominate, while the binding of haemopexin by HxuA relies more on polar and charged interactions.

Both direct extraction and haemophore-employing strategies require the removal of haem from a high affinity-binding site either on the haemophore or host protein. Structures elucidated recently now provide at least partial descriptions as to how haem can be removed from a haemophore (HasA) or from haemoglobin or haemopexin. A common theme in the receptor-mediated acquisition of haem from haemophore HasA, haemopexin and haemoglobin is the dynamics of the target protein upon binding to the receptor. This is most evident in the HxuA-haemopexin example, where a whole domain of haemopexin is presumably displaced, but also more subtly in other examples where backbone movements of the target proteins lead to haem ligands being displaced or haem binding pockets being opened up. Interestingly, both the removal of haem from haemopexin and HasA additionally use steric hindrance to displace the haem group from its high-affinity binding site, while no steric clashes are observed in the LsdH-haemoglobin structures. It remains to be seen if this is due to the mutations used to trap the complex generating an incomplete picture or, perhaps more plausibly, that haem removal here is facilitated by disrupting the haem binding pocket on haemoglobin and supplying a nearby haem-binding site to accept haem after it dissociates. It is currently unknown how systems employing TonB dependent receptors remove haem from haemoglobin and whether these share a common mechanism, as suggested by an apparent pervasive role of the plug loop, or, as suggested by their limited sequence conservation, they have evolved distinct methods.

An interesting observation in comparing HpuAB system with LsdH is the interaction of the non-haem-binding (HpuA and LsdH<sup>N2</sup>) moiety

with haemoglobin. Both HpuA and LsdH<sup>N2</sup> bind between the A and E helices of haemoglobin albeit HpuA interacting with the beta chain and LsdH<sup>N2</sup> with the alpha chain. While it could be ventured that binding at this site has long distance effects on the stability of haem binding, no change in the haem binding site is observed upon binding and it could rather be simply a convenient place to capture haemoglobin due to the exposure of a patch of hydrophobic surface. A more speculative explanation could be that binding at this site is only possible to haem-bound haemoglobin and that structural change to this site of haemoglobin following haem removal leads to dissociation. This explanation would be consistent with the observed effect of HpuA on haemoglobin dissociation [28].

Arguably the most crucial open question is whether we can use this meticulously garnered knowledge to good effect in the development of novel, clinically useful therapeutics against haem-dependent pathogens. *Haemophilus influenza* is a haem-auxotroph (lacking a haem biosynthesis pathway) and yet requires haem for growth [86], implying small molecules that interfere with its haem uptake pathways could be bactericidal. Structural knowledge of the proteins and complexes is important for rational design of such inhibitors. Even for pathogens that do not have an absolute haem requirement, or can generate their own haem, blocking their haem acquisition systems could hinder growth. Alternatively, toxic non-iron porphyrins have long been touted as potential antimicrobials hypothesised to enter the cell via haem import receptors in a Trojan-horse manner [87,88]. Additionally, as part of extracellular systems, the proteins involved in haem uptake are potential vaccine candidates. In using a haemoprotein receptor as a vaccine antigen, it would be beneficial to prevent it binding to its host target protein to allow antibodies to access a full range of epitopes and to increase the chance that antibodies raised will block the functional host-pathogen interaction. Structural understanding of receptor-host protein complexes will be key to designing mutations facilitating this.

## Conflicts of interests

The author declares no conflicts in financial interests.

## Transparency document

The Transparency document associated with this article can be found, in the online version.

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